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In vitro metabolic studies using homogenized horse liver in place of horse liver microsomes

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The study of the metabolism of drugs, in particular steroids, by both *in vitro* and *in vivo* methods has been carried out in the authors' laboratory for many years. For *in vitro* metabolic studies, the microsomal fraction isolated from horse liver is often used. However, the process of isolating liver microsomes is cumbersome and tedious. In addition, centrifugation at high speeds (over 100 000 g) may lead to loss of enzymes involved in phase I metabolism, which may account for the difference often observed between *in vivo* and *in vitro* results. We have therefore investigated the feasibility of using homogenized horse liver instead of liver microsomes with the aim of saving preparation time and improving the correlation between *in vitro* and *in vivo* results. Indeed, the preparation of the homogenized horse liver was very simple, needing only to homogenize the required amount of liver. Even though no further purification steps were performed before the homogenized liver was used, the cleanliness of the extracts obtained, based on gas chromatography-mass spectrometry (GC-MS) analysis, was similar to that for liver microsomes. Herein, the results of the *in vitro* experiments carried out using homogenized horse liver for five anabolic steroids – turinabol, methenolone acetate, androst-4-ene-3,6,17-trione, testosterone, and epitestosterone – are discussed. In addition to the previously reported *in vitro* metabolites, some additional known *in vivo* metabolites in the equine could also be detected. As far as we know, this is the first report of the successful use of homogenized liver in the horse for carrying out *in vitro* metabolism experiments.

Keywords: homogenized horse liver; *in vitro*; metabolism; microsomes; steroids

Introduction

The study of the metabolism of drugs by *in vivo* and *in vitro* methods provides vital information for laboratories involved in doping control testing in sports. This is especially important if the parent drug is quickly eliminated, making the detection of metabolites desirable. Such studies are particularly useful for the metabolism of steroids in which a large number of biotransformations is possible leading to many plausible metabolites. Without information from these *in vivo* or *in vitro* studies, it would be very difficult to identify the most appropriate analytes for controlling the misuse of a particular steroid. Ideally, *in vivo* studies are preferred to *in vitro* experiments since the former would give the best representation of the metabolism of a drug observed in a particular species. *In vivo* studies also give pharmacokinetic data so that the analyte(s) with the longest detection time can be targeted. However, there are practical implications in arranging *in vivo* studies, such as the availability of the animal or suitable resources. There are also ethical issues, especially when drugs without toxicology profiles, for example 'designer' drugs, need to be studied. *In vitro* experiments overcome these problems and can be an attractive alternative: less time-consuming to set up, require fewer resources, and give incubation mixtures that are much simpler to work with than equine biological fluids. However, the major concern for carrying out *in vitro* experiments is how well the results correlate with those from *in vivo* studies. *In vitro* experiments have been used to complement *in vivo* studies in the authors' laboratory for many years to study the metabolism of anabolic steroids in horses including oxymetholone,[1] mestanolone,[1] danazol,[2] methenolone acetate,[3] clostebol acetate,[4] turinabol,[5] mesterolone,[6] and androst-4-ene-3,6,17-trione.[7] However, there were metabolites detected *in vivo* that could not be detected in the *in vitro* experiments and in some cases, these were the metabolites with the longest detection times. The sometimes poor representation obtained is a major reason why *in vitro* studies have not become common practice.[8]

Various liver fractions can be used for performing *in vitro* experiments, including liver microsomes, liver cytosol, or S9 fractions.[9] Liver microsomes mainly contain enzymes involved in phase I metabolism, whereas the cytosol fraction contains enzymes for phase II metabolism. The S9 fraction contains both the microsomes and cytosol fractions. The authors' laboratory uses horse liver microsomes isolated from fresh horse liver after two stages of centrifugation. However, the process is cumbersome. Fresh liver has to be first cut into small pieces and homogenized before centrifugation at high speeds (over 100 000 g), with all steps and reagents kept at 4 °C. Once isolated, however, the liver microsomes can be used for at least two years if stored at −80 °C immediately after preparation and before use. In order to obtain a reasonable amount of microsomes from the horse liver whilst still fresh, i.e. within the day of receipt, the preparation steps need to be repeated many times, making the whole process very tedious. In addition, centrifugation is not a very selective process and inevitably, some enzymes involved in phase I metabolism will be lost in the process. In an attempt to improve the correlation

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between *in vitro* and *in vivo* results, we have investigated the use of homogenized liver instead of liver microsomes. The preparation of homogenized liver is very simple. The fresh liver needs only to be cut into thin slices and stored at −80 °C immediately after receipt. The required amount of frozen liver is then homogenized in the incubation reagent at 4 °C immediately prior to the *in vitro* experiment. *In vitro* experiments are more commonly carried out using liver fractions; however, the use of homogenized liver without centrifugation has been previously reported in rat,[10–17] human,[18,19] and fish.[20] It must be noted, however, that the most recent report of the use of homogenized liver was in 2002.[17] Nevertheless, there has been no previous report comparing the results of *in vitro* experiments using either liver microsomes or homogenized liver. Thus, to our knowledge, this is the first report of such a study and certainly, the first report of the use of homogenized liver from horses.

This paper describes the results of experiments with homogenized liver, using turinabol, methenolone acetate, androst-4-ene-3,6,17-trione, testosterone, and epitestosterone as model substrates.

Materials and methods

Materials

Turinabol was obtained from Hunan Steroid Chemicals Co. Ltd (Hunan, China). Methenolone acetate was obtained from Shanghai Freeman (Shanghai, China). Androst-4-ene-3,6,17-trione, 5α-androstane-3β,17α-diol, 5α-androstane-3α,17α-diol, 5α-androstane-3α,17β-diol, 5β-androstane-3α,17α-diol and 5β-androstane-3,17-dione were obtained from Steraloids (Rhode Island, USA). Testosterone was purchased from the United States Pharmacopeia (Rockville, MD, USA). Androsterone was obtained from Alltech (Deerfield, IL, USA). Epitestosterone, androstenedione, 5α-androstane-3β,17β-diol, 5β-androsterone, 3-epiandrosterone, β-nicotine adenine dinucleotide (β-NAD), glucose-6-phosphate, magnesium chloride ($MgCl_2$), glucose-6-phosphate dehydrogenase, Tris (TRIZMA®), dithioerythritol (DTE), ammonium iodide (NH_4I) and pyridine were obtained from Sigma-Aldrich (St Louis, MO, USA). LiChrosolv® grade *n*-heptane was purchased from Merck (Darmstadt, Germany). Ethyl acetate, potassium chloride and sodium dihydrogen phosphate were all of GR grade from Merck. Anhydrous sodium sulfate (AR grade) was obtained from Uni-Chem (Karlsruhe, Germany). Pentafluoropropionic acid anhydride (PFPA) was obtained from Pierce (IL, USA). *N*-Methyl-*N*-trimethylsilylfluoroacetamide (MSTFA) and *N,O-bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) with 5% trimethylchlorosilane (TMCS) were obtained from Thermo Scientific (IL, USA). Acetic anhydride was obtained from International Laboratory Limited (San Bruno, CA, USA). Deionized water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

Preparation of horse liver microsomes

Horse liver microsomes were isolated from fresh horse liver supplied by the Equine Hospital of The Hong Kong Jockey Club. Fresh liver was obtained immediately post mortem from a 5-year-old gelding (488 kg) diagnosed with osteoarthritis. Small pieces of horse liver were homogenized in Tris/KCl buffer (0.05 M, pH 7.4). The homogenate was centrifuged at 10 000 g for 25 min, and the microsomes were isolated from the resulting supernatant by centrifugation at 105 000 g for 1 h. The pellet of microsomes was then washed twice with Tris/KCl buffer (0.05 M, pH 7.4). All the preparation steps and the isolated microsomes were conducted or kept at 4 °C. Once isolated, the horse liver microsomes were stored immediately at −80 °C as 30 µl aliquots.

Preparation of homogenized horse liver

Fresh horse liver (from the same horse that provided the horse liver microsomes in the present study) was cut into 1-cm thick slices and stored immediately at −80 °C. When required, the frozen horse liver was further cut into 1-cm cube pieces and homogenized at 4 °C in 2 ml of the incubation reagent.

Incubation studies using liver microsomes and homogenized liver

Horse liver microsomes (30 µl) or homogenized liver (from 1-cm cube) and 2 ml of β-NAD (1.5 mM), glucose-6-phosphate (7.5 mM), $MgCl_2$ (4.5 mM), glucose-6-phosphate dehydrogenase (1 U/ml) and sodium dihydrogen phosphate solution (50 mM; pH 7.4) were incubated with the drug under study (0.5 mg) at 37 °C for 2 h with shaking. The reaction was terminated by heating at 100 °C for 10 min. The mixture was centrifuged at 2100 g for 10 min, and the supernatant was extracted twice with ethyl acetate (5 ml), and the extract was evaporated to dryness. The dried residue was derivatized (by trimethylsilylation or acylation) for gas chromatography-mass spectrometry (GC-MS) analysis. Control experiments in the absence of either (1) the drug or (2) liver microsomes or homogenized liver were performed in parallel.

Derivatization for GC-MS analysis

Trimethylsilyl derivatives were prepared by either adding MSTFA/NH_4I/DTE (1000 : 2:4, *v/w/w*, 30 µL) or BSTFA (30 µL) to the dried residue and incubated at 60 °C for 15 min. The resulting solution was injected directly into the GC-MS.

Acetylated derivatives were prepared by adding acetic anhydride (50 µl) and dry pyridine (100 µl) to the dried residue, followed by incubation at 60 °C for 30 min. The solvent was evaporated to dryness and the dry residue was reconstituted with ethyl acetate (30 µl) before analysis by GC-MS.

Pentafluoropropionyl (PFP) derivatives were prepared by adding acetonitrile (100 µl) and PFPA (30 µl) to the dry residue. The mixture was incubated at 60 °C for 15 min and then evaporated to dryness at 60 °C under nitrogen. The residue was reconstituted with *n*-heptane (30 µl). The resulting solution was analysed by GC-MS.

Instrumentation

An Agilent 6890N Network GC system coupled to an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, California, USA) was used. Separation was performed on an HP-1 MS (~30 m × 0.25 mm, 0.25 µm film thickness) column with a constant helium flow of 1.2 ml/min. The oven temperature was set initially at 110 °C for 1 min, increased to 150 °C at 60 °C/min and then to 320 °C at 15 °C/min, and finally held at 320 °C for 4 min. Samples (1 µl) were injected at 260 °C in splitless mode. All GC-MS analyses were performed in the EI mode with full-scan acquisition (110–630 amu).

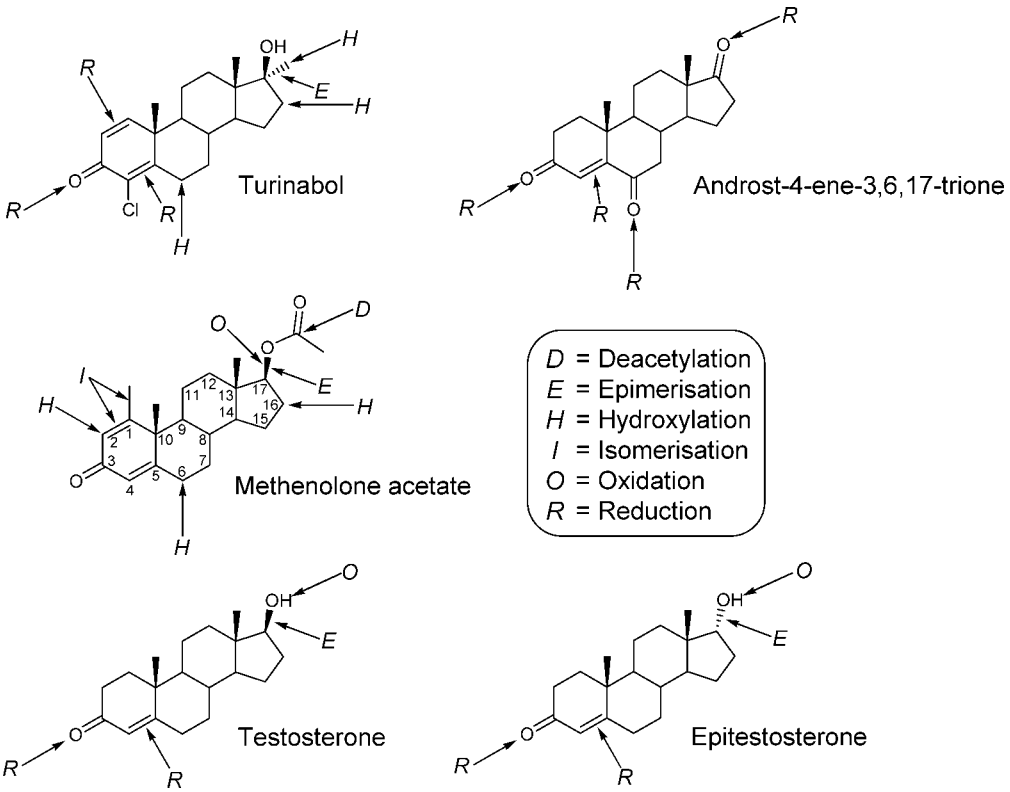

Figure 1. A summary of biotransformations observed *in vivo* and *in vitro* for turinabol, methenolone acetate, androst-4-ene-3,6,17-trione, testosterone and epitestosterone.

Results and discussion

The procedure for carrying out *in vitro* experiments using horse liver microsomes have been in use by the authors' laboratory for many years and successfully applied to a number of anabolic steroids, including oxymetholone,[1] mestanolone,[1] danazol,[2] methenolone acetate,[3] clostebol acetate,[4] turinabol,[5] mesterolone,[6] and androst-4-ene-3,6,17-trione.[7] Of these, turinabol, methenolone acetate and androst-4-ene-3,6,17-trione were selected for the present study using homogenized liver since their metabolites have been well studied and previously reported (Figure 1). Testosterone and epitestosterone have also been included in this present study due to the marked difference in results between the use of liver microsomes and homogenized liver. A summary of the *in vivo* metabolites reported previously, together with the *in vitro* results using liver microsomes and homogenized liver, is shown in Table 1. It was calculated that the 1-cm cube liver used is equivalent to 30 µl of horse liver microsomes. The *in vitro* incubation studies were all carried out with control experiments in the absence of either (1) the drug or (2) liver microsomes or homogenized liver in parallel. For all steroids, the control experiments without the drug added did not give rise to the parent drug or any of the corresponding metabolites. Correspondingly, the control experiments without the liver microsomes or homogenized liver only gave rise to the parent drug and none of the metabolites.

Turinabol

Incubation of turinabol in the presence of liver microsomes has previously been reported to give five metabolites resulting from hydroxylation at C6, C16, and C20 (T1-T5).[5] In the present study, the use of both liver microsomes and homogenized liver yielded only four of the five metabolites (T1-T3 and T5). The absence of metabolite T4 may be due to the use of a different liver compared to the study carried out previously (Figure 2). In order to minimize the problem of individual variation, future *in* vitro studies will be carried out by pooling liver from two or more horses. Much the same as administration studies where it is usual practice to include results from more than one horse, *in vitro* studies should also be carried out in the same manner. Of the four metabolites detected, metabolites T2 and T5, namely, 20-hydroxyturinabol and 6β,20-dihydroxyturinabol are also *in vivo* metabolites. However, seven other *in vivo* metabolites, i.e. 17-epiturinabol (T6) and six others from A-ring reduction and D-ring hydroxylation (three stereoisomers each for 4-chloro-17-methyl-androstene-3,17,20-triol (T7-T9) and 4-chloro-17-methyl-androstene-3,16,17-triol (T10-T12)), were not detected in the *in vitro* experiments.

The use of crude homogenized liver in place of liver microsomes obtained from liver after two stages of centrifugation naturally raised concern about the cleanliness of the extract obtained for analysis. The total ion chromatograms (TIC) after trimethylsilylation of the extracts from incubation of turinabol with liver microsomes and homogenized liver are shown in Figure 2. The number of peaks and their relative abundance were very similar in both cases, thus the use of homogenized liver did not seem to yield additional matrix peaks which could interfere with metabolite identification. In fact, the centrifugation step post-incubation proved to be sufficient to remove all the liver tissue debris. A closer examination of Figure 2 shows that the relative abundance of the *in vitro* metabolites is comparable after both incubations. Although no additional *in vivo* metabolites were obtained after incubation

Table 1. Metabolites obtained *in vivo* and *in vitro* after incubation using horse liver microsomes and homogenized horse liver for turinabol, methenolone acetate, androst-4-ene-3,6,17-trione, testosterone and epitestosterone

Metabolites	Biotransformation from parent drug*	*In vivo*	*In vitro*	
			Using microsomes	Using liver
Turinabol				
6β-Hydroxyturinabol (**T1**)	H	×	√	√
20-Hydroxyturinabol (**T2**)	H	√	√	√
6β,16α-Dihydroxyturinabol (**T3**)	H	×	√	√
6β,16β-Dihydroxyturinabol (**T4**)	H	×	×#	×
6β,20-Dihydroxyturinabol (**T5**)	H	√	√	√
17-Epiturinabol (**T6**)	E	√	×	×
4-Chloro-17-methyl-androstene-3,17,20-triol (**T7-T9**)	H & R	√	×	×
4-Chloro-17-methyl-androstene-3,16,17-triol (**T10-T12**)	H & R	√	×	×
Methenolone acetate				
1-Methyl-5α-androst-1-en-17β-ol-3-one (**M1**)	D	√	√	√
1-Methyl-5α-androst-1-ene-3,17-dione (**M2**)	D & O	×	√	√
1-Methyl-5α-androst-1-en-6-ol-3,17-dione (**M3**)	D & O & H	×	√	√
1-Methylen-5α-androstan-2-ol-3,17-dione (**M4** & **M5**)	D & O & H & I	×	√	√
1-Methyl-5α-androst-1-en-16-ol-3,17-dione (**M6**)	D & H & O	√	√	√
1-Methyl-5α-androst-1-ene-16β,17β-diol-3-one (**M7**)	D & H	√	√	√
1-Methyl-5α-androst-1-ene-16α,17β-diol-3-one (**M8**)	D & H	√	√	√
1-Methyl-5α-androst-1-ene-16β,17α-diol-3-one (**M9**)	D & H	√	×	√
1-Methyl-5α-androst-1-ene-16α,17α-diol-3-one (**M10**)	D & H	√	×	√
1-Methyl-5α-androst-1-en-17α-ol-3-one (**M11**)	D & E	√	×	√
Androst-4-ene-3,6,17-trione				
6α-Hydroxyandrost-4-ene-3,17-dione (**A1**)	R	√	√	√
3-Hydroxyandrost-4-ene-6,17-dione (**A2** & **A3**)	R	√	√	√
6α,17β-Dihydroxyandrost-4-en-3-one (**A4**)	R	√	×	√
6,17-Dihydroxyandrost-4-en-3-one (**A5** & **A6**)	R	√	×	√
3β,6β-Dihydroxyandrost-4-en-17-one (**A7**)	R	√	×	×
3,6-Dihydroxyandrost-4-en-17-one (**A8**)	R	√	×	×
3,6-Dihydroxyandrostan-17-one (**A9**)	R	√	×	×
3,17-Dihydroxyandrostan-6-one (**A10**)	R	√	×	√
Testosterone				
Androstenedione (**Te1**)	O	×	√	√
Epitestosterone (**Te2**)	E	×	×	√
5α-Androstane-3β,17α-diol (**Te3**)	R	√	×	√
5α-Androstane-3β,17β-diol (**Te4**)	R	√	×	√
5α-Androstane-3α,17α-diol (**Te5**)	R	×	×	√
5α-Androstane-3α,17β-diol (**Te6**)	R	×	×	√
5β-Androstane-3α,17α-diol (**Te7**)	R	×	×	√
5β-Androstane-3,17-dione (**Te8**)	O & R	×	×	√
Androsterone (**Te9**)	O & R	×	×	√
5β-Androsterone (**Te10**)	O & R	×	×	√
3-Epiandrosterone (**Te11**)	O & R	√	×	√
Epitestosterone				
Androstenedione (**E1**)	O	–	√	√
Testosterone (**E2**)	E	–	×	√
5α-Androstane-3β,17α-diol (**E3**)	R	–	×	×
5α-Androstane-3β,17β-diol (**E4**)	R	–	×	×
5α-Androstane-3α,17α-diol (**E5**)	R	–	×	√
5α-Androstane-3α,17β-diol (**E6**)	R	–	×	×
5β-Androstane-3α,17α-diol (**E7**)	R	–	×	√
5β-Androstane-3,17-dione (**E8**)	O & R	–	×	√
Androsterone (**E9**)	O & R	–	×	√
5β-Androsterone (**E10**)	O & R	–	×	√
3-Epiandrosterone (**E11**)	O & R	–	×	√

* *D = Deacetylation*; *E = Epimerisation*; *H = Hydroxylation*; *I = Isomerisation*; *O = Oxidation*; *R = Reduction*.
# Observed previously[5].

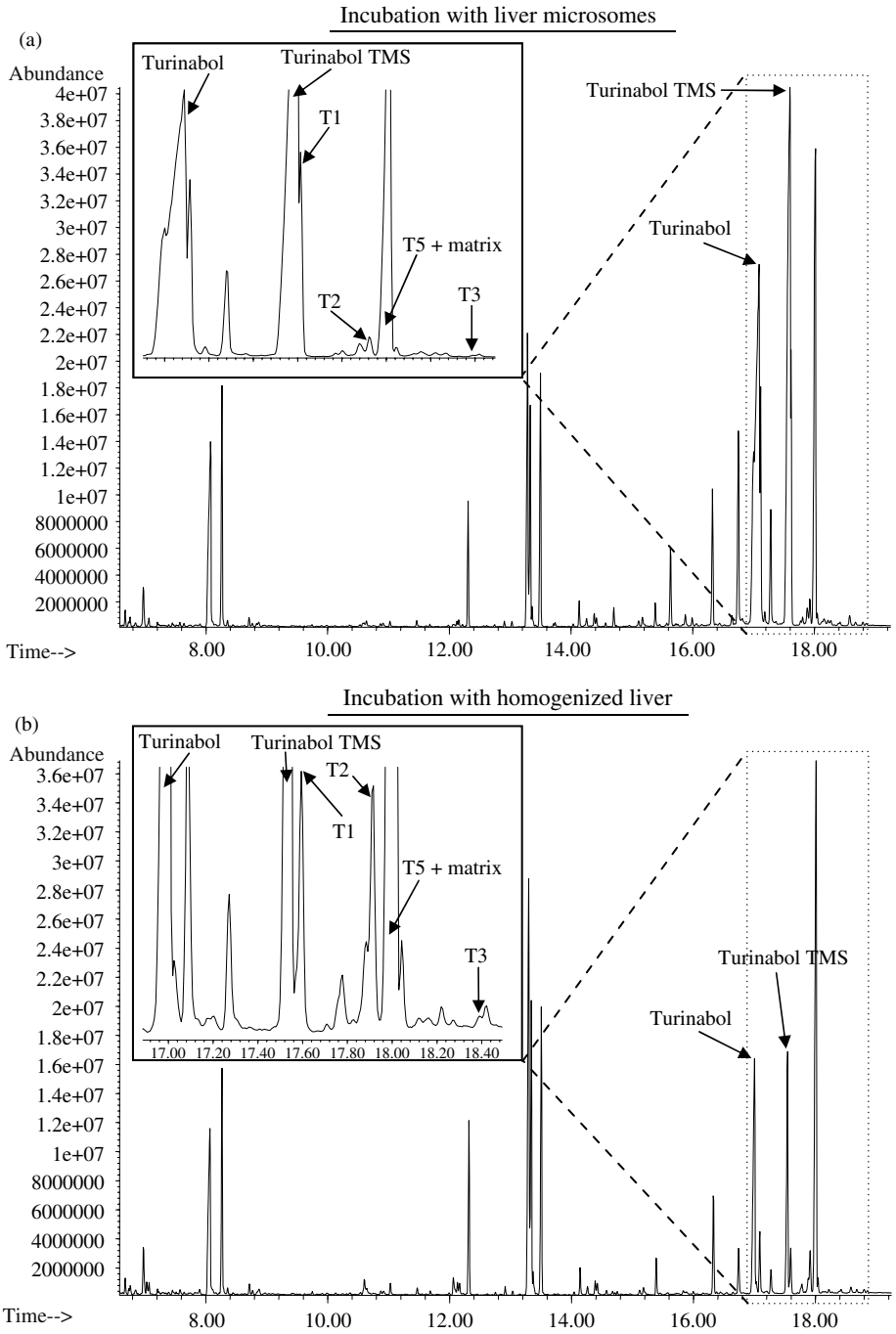

Figure 2. The total ion chromatograms obtained *in vitro* after incubation of turinabol using (a) horse liver microsomes and (b) homogenized horse liver.

using homogenized liver, it has been clearly demonstrated that the extract obtained is no dirtier (no additional major matrix peaks) than using liver microsomes.

Methenolone acetate

For methenolone acetate, previously, eight *in vitro* metabolites (M1-M8) were reported after incubation with liver microsomes,[3] and in the present study, an additional three *in vivo* metabolites, namely M9-M11 were obtained using homogenized liver. Metabolites M9 and M10 are stereoisomers of M6 and M7 with the hydroxyl group at C17 being 17α instead of 17β. Metabolite M11 is 17-epimethenolone, also containing a 17α-hydroxy group. One probable route to the formation of metabolites M9-M11 is *via* epimerisation of the 17β-hydroxy group in M6, M7 and methenolone respectively. Since M9-M11 were all detected after incubation with homogenized liver but not with liver microsomes, this suggests that the epimerization observed may be the result of enzymatic action, with the enzymes responsible for epimerization lost during the isolation of liver microsomes. If isolated, the epimerization enzymes are probably located in the cytosol fraction. Epimerization at C17 was not observed *in vitro* for turinabol, although 17-epiturinabol is a reported *in vivo* metabolite. This is, however, not surprising as the *in vivo* 17-epimerization of

17β-hydroxy-17α-methyl steroids have been shown to occur *via* the spontaneous degradation of their corresponding 17-sulfate conjugates.[21] Since only phase I metabolites have been investigated for this present study, it is reasonable that 17-epiturinabol was not detected as an *in vitro* metabolite.

Androst-4-ene-3,6,17-trione

In the case of androst-4-ene-3,6,17-trione, of the ten metabolites detected *in vivo*, only three were detected *in vitro* using liver microsomes.[7] These were reduction of the keto group at C6 to give 6α-hydroxyandrost-4-ene-3,17-dione (A1) and reduction of the C3 keto group to give 3α and 3β-hydroxyandrost-4-ene-6,17-dione (A2 and A3). The use of homogenized liver yielded an additional four *in vivo* metabolites, A4-A6 and A10, all involving reduction of the 17-keto group. Of these, 3,17-dihydroxyandrostan-6-one (A10) was previously reported to be the metabolite with the longest detection time (approx. 2 days). The metabolite A6, 6,17-dihydroxyandrost-4-en-3-one, with the second longest detection time (approx. 1 day), was also detected. The *in vivo* metabolites with reduction at both C3 and C6 (A7-A9) were, however, not observed with homogenized liver.

Testosterone

Testosterone has previously been reported to give three major *in vivo* metabolites in horses,[22,23] namely, 5α-androstane-3β,17α-diol, 5α-androstane-3β,17β-diol, and 5α-androstan-3β-ol-17-one (3-epiandrosterone). All three metabolites were observed *in vitro* using homogenized liver but were not detected using liver microsomes. In fact, the only *in vitro* metabolite obtained using liver microsomes was androstenedione (Te1), from oxidation of the 17β-hydroxy to a keto group. Incubation using homogenized liver also gave androstenedione as the major metabolite. The other major *in vitro* metabolite obtained was epitestosterone (Te2), giving another example of the presence of epimerase enzymes in the homogenized liver that is absent in the liver microsomes. For the androstanediols, although only two of the four isomers of 5α-androstanediols, i.e. the 3β-hydroxy isomers (Te3 and Te4), were obtained *in vivo*, all four isomers (Te3-Te6) were present after incubation with homogenized liver. In addition, another stereoisomer of androstanediol, 5β-androstane-3α,17α-diol (Te7), was detected along with four other metabolites (Te8-Te11), giving a total of 11 *in vitro* metabolites obtained using homogenized liver compared to only one *in vitro* metabolite using liver microsomes.

Epitestosterone

Although no previous *in vivo* studies have been reported for epitestosterone, the *in vitro* results were very similar to testosterone. Again only one metabolite, androstenedione, was detected using liver microsomes. For homogenized liver, the major metabolite found was also androstenedione with the second major metabolite being testosterone, resulting from epimerization of epitestosterone. Of the 11 *in vitro* metabolites of testosterone detected using homogenized liver, eight (including the corresponding 17-epimer) were obtained for epitestosterone. The three metabolites that could not be detected were three isomers of 5α-androstanediols, with only 5α-androstane-3α,17α-diol detected.

In vitro experiments performed using liver that has been stored at $-80\,^{\circ}C$ immediately upon receipt for over half a year has consistently given the same results. This demonstrates that liver can be safely stored in this way with no obvious loss in activity.

Conclusion

This is the first report of the successful use of homogenized liver in place of liver microsomes for carrying out *in vitro* metabolism experiments in the horse. Using homogenized liver eliminates the need to carry out the cumbersome and tedious isolation of liver microsomes, saving much preparation time. In addition, the extract obtained was no dirtier based on GC-MS analysis. More importantly, additional *in vivo* metabolites were obtained after incubation using homogenized liver. For methenolone acetate and androst-4-ene-3,6,17-trione, respectively, three and four additional *in vivo* metabolites were detected *in vitro* using homogenized liver compared to liver microsomes. In the case of androst-4-ene-3,6,17-trione, the additional *in vivo* metabolites detected using homogenized liver also included the metabolite with the longest detection time after administration. For testosterone, all three previously reported *in vivo* metabolites were detected *in vitro* using homogenized liver, whereas none of the *in vivo* metabolites were detected using liver microsomes. This new approach of using homogenized liver in place of liver microsomes for *in vitro* studies has also been successfully extended to other classes of drugs besides steroids and has proven to work well. Future work would include the investigation of phase II metabolism. It is envisaged that phase II metabolites may be easily obtained in this way.

A recent change in ILAC-G7 *Accreditation Requirements and Operating Criteria for Horseracing Laboratories*[24] has allowed the use of metabolites obtained from *in vitro* experiments as reference materials. The use of homogenized liver for the *in vitro* synthesis of drug metabolites would therefore be a useful and convenient approach in obtaining reference materials which are not available commercially.

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References

[1] P. W. Tang, W. C. Law, T. S. M. Wan, D. L. Crone. Metabolic studies of some oral anabolic steroids in horses: Part 1. oxymetholone and mestanolone, in *Proceedings of the 12th International Conference of Racing Analysts and Veterinarians,* (Eds: B. Laviolette, M. R. Koupai-Abyazani), R&W Publications: Newmarket, UK, **2000**, pp. 118–124.

[2] P. W. Tang, K. L. Watkins, T. S. M. Wan. Metabolic studies of oral anabolic steroids in horses. Part 2: Danazol, in *Proceedings of the 13th International Conference of Racing Analysts and Veterinarians,* (Eds: R. B. Williams, E. Houghton, J. F. Wade), R&W Publications: Newmarket, UK, **2001**, pp. 171–178.

[3] E. N. M. Ho, D. K. K. Leung, T. S. M. Wan, N. H. Yu. Metabolic studies of methenolone acetate in horses. *Anal. Chim. Acta* **2005**, *540*, 111.

[4] G. N. W. Leung, E. N. M. Ho, D. K. K. Leung, F. P. W. Tang, T. S. M. Wan, J. H. K. Yeung, H. N. C. Wong. Metabolic studies of clostebol acetate in horses. *Chromatographia*, **2005**, *61*, 397.

[5] E. N. M. Ho, W. H. Kwok, D. K. K. Leung, T. S. M. Wan, A. S. Y. Wong. Metabolic studies of turinabol in horses. *Anal. Chim. Acta* **2007**, *586*, 208.

[6] E. N. M. Ho, D. K. K. Leung, G. N. W. Leung, T. S. M. Wan, H. N. C. Wong, X. Xu, J. H. K. Yeung. Metabolic studies of mesterolone in horses. *Anal. Chim. Acta* **2007**, *596*, 149.

[7] G. N. W. Leung, F. P. W. Tang, T. S. M. Wan, C. H. F. Wong, K. K. H. Lam, B. D. Stewart. *In vitro* and *in vivo* studies of androst-4-ene-3,6,17-trione in horses by gas chromatography-mass spectrometry. *Biomed. Chromatogr.* **2010**, *24*, 744.

[8] J. P. Scarth, H. A. Spencer, S. E. Timbers, S. C. Hudson, L. L. Hillyer. The use of *in* vitro technologies coupled with high resolution accurate mass LC-MS for studying drug metabolism in equine drug surveillance. *Drug Testing Anal.* **2010**, *2*, 1.
[9] E. F. A. Brandon, C. D. Raap, I. Meijerman, J. H. Beijnen, J. H. M. Schellens. An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol. Appl. Pharmacol.* **2003**, *189*, 233.
[10] M. M. Asaad, H. Barry, D. E. Clarke, B. N. Dixit. Effect of ethanol on the oxidative metabolism of tryptamine by rat liver homogenate. *Brit. J. Pharmacol.* **1974**, *50*, 277.
[11] H. Shah, S. P. Hartman, S. Weinhouse. Formation of carbonyl chloride in carbon tetrachloride metabolism by rat liver *in vitro*. *Cancer Res.* **1979**, *39*, 3942.
[12] G. C. Bolton, L. A. Griffiths. The metabolic disposition of [^{14}C]pivhydrazine, [^{14}C]mebanazine, and [^{14}C]benzylhydrazine in the rat. *Drug Metab. Dispos.* **1979**, *7*, 388.
[13] J. Tjørnelund, S. H. Hansen, C. Cornett. New metabolites of the drug 5-aminosalicylic acid. I: *N*-β-D-glucopyanosyl-5-aminosalicylic acid. *Xenobiotica* **1989**, *19*, 891.
[14] K. Shanmuganathan, T. Koudriakova, S. Nampalli, J. Du, J. M. Gallo, R. F. Schinazi, C. K. Chu. Enhanced brain delivery of an anti-HIV nucleoside 2′-F-ara-ddI by xanthine oxidase mediated biostransformation. *J. Med. Chem.* **1994**, *37*, 821.
[15] R. A. Newman, A. Fuentes, J. M. Covey, J. A. Benvenuto. Preclinical pharmacology of the natural marine product dolastatin 10 (NSC 376128). *Drug Metab. Dispos.* **1994**, *22*, 428.
[16] R. M. Crooke, M. J. Graham, M. J. Martin, K. M. Lemonidis, T. Wyrzykiewiecz, L. L. Cummins. Metabolism of antisense oligonucleotides in rat liver homogenates. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 140.
[17] A. Marwah, P. Marwah, H. Lardy. Ergosteroids VI. Metabolism of dehydroepiandrosterone by rat liver in vitro: a liquid chromatographic-mass spectrometric study. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2002**, *767*, 285.
[18] D. N. Bailey. Cocapropylene (propylcocaine) formation by human liver in vitro. *J. Anal. Toxicol.* **1995**, *19*, 1.
[19] S. Ammon, O. von Richter, U. Hofmann, K. P. Thon, M. Eichelbaum, G. Mikus. In vitro interaction codeine and diclofenac. *Drug Metab. Dispos.* **2000**, *28*, 1149.
[20] A. Machon, M. J. North, N. C. Price, D. E. Wells. The *in vitro* metabolism of Eulan WA New by liver homogenates from freshwater fish. *Comp. Biochem. Physiol. C* **1986**, *84C*, 113.
[21] W. Schänzer, G. Opfermann, M. Donike. 17-Epimerization of 17α-methyl anabolic steroids in humans: metabolism and synthesis of 17α-hydroxy-17β-methyl steroids. *Steroids* **1992**, *57*, 537.
[22] E. Houghton, M. C. Dumasia. Studies related to the metabolism of anabolic steroids in the horse: testosterone. *Xenobiotica* **1979**, *9*, 269.
[23] E. Houghton, Anabolic steroids in the horse – a review of current knowledge, in *Proceedings of the 9th International Conference of Racing Analysts and Veterinarians,* (Ed: C. R. Short), R&W Publications: New Orleans, **1992**, pp. 3–16.
[24] ILAC-G7 : 6/2009. *Accreditation Requirements and Operating Criteria for Horseracing Laboratories*. Available at: http://www.ilac.org/documents/ILAC_G7_06_2009.pdf.